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FURTHER OBSERVATIONS ON EUGLENA AND B_{12}[1]

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In a previous paper (Robbins, Hervey and Stebbins 1950) from this laboratory, methods for using *Euglena gracilis* var. *bacillaris* as a means of bioassay for vitamin B_{12} were described and some results cited. The present paper reports an extension of the earlier work.

Methods. The methods followed were essentially those described in the earlier paper. Vitamin B_{12} was determined using optical density as a measure of growth of *Euglena* in medium I (acid). When turbidity or color of the solutions analyzed interfered or where growth was slight, counts of *Euglena* per ml. were substituted for optical density measurements. The filter paper disc (FPD) method of estimating B_{12} was used also though the major dependence was placed on growth in liquid media because of the greater sensitivity.

In the main *Euglena* has proved in our hands useful for the detection and estimation of B_{12} or its *Euglena* equivalent in extracts of natural materials; this is contrary to the experience of Picken and Bauriedel (1950). However, a number of factors must be considered in evaluating results.

Glassware. For cleaning glassware we have used chromic acid cleaning mixture followed by thorough rinsing with tap and distilled water. This treatment removes residual B_{12} and we have not detected deleterious results from the chromic acid, (Richards 1936). On the other hand, tubes which had contained 50 or 100 $\mu\mu$g. of B_{12} per ml. were washed with soap, water and a test tube brush and rinsed 3 or 4 times with tap water and 3 or 4 times with distilled water. From 1 to 3 per cent of the B_{12} remained in the tubes (Loy, Haggerty and Kline 1950).

Inhibitory material. Extracts of natural materials may contain inhibitory substances as well as vitamin B_{12}. Some extreme examples which were detected by the FPD method were cited in our earlier paper. Deleterious substances may be present in amounts which reduce growth but do not inhibit it entirely. Determinations of B_{12} for such extracts in which *Euglena* is grown in liquid media and the optical density is measured, will be low; with the FPD method they may be high. The presence of inhibitory material may be detected and the effect eliminated in many instances by comparing assays on a series of dilutions.

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Variability of Euglena. As described earlier (Robbins, Hervey and Stebbins 1950), our stock *Euglena* is maintained in the light of 25° C. in 25 ml. quantities of liquid medium I (acid) containing 50 $\mu\mu$g. of B_{12} per ml. Transfers are made every 2 to 4 days. Under such conditions a culture of sufficient density normally develops in 2 or 3 days to give a reading on a Klett-Summerson photoelectric colorimeter of 50 to 55 when diluted 5 ×.

In early December 1950 our standard cultures became pale green instead of dark green and it was necessary to supplement a 3 or 4 day old culture with older cultures to obtain sufficient density for the standard

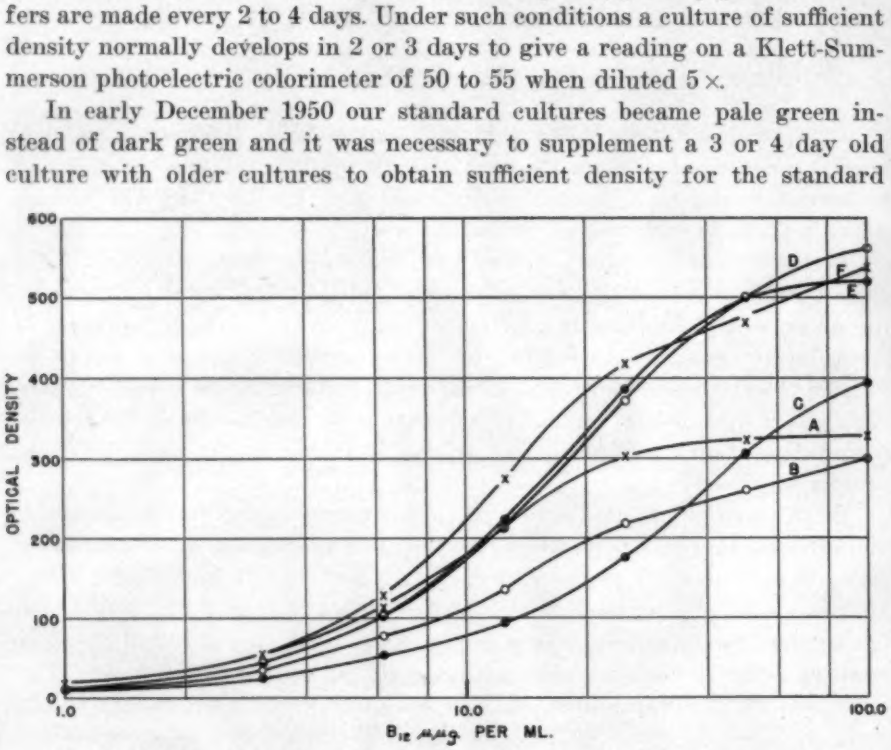

FIG. 1. Optical density of *Euglena* grown 7 days in solution containing crystalline B_{12} Curve A, inoculated 12/13/50; B, 12/15; C, 12/29 with pale green culture of *Euglena*. Curve D, solutions inoculated 1/5/51; E, 1/8 and F, 1/26 with culture which had spontaneously returned to dark green condition.

inoculum. The B_{12} standard curves obtained with this inoculum were low with maxima 1/2 to 4/5 those normally observed (fig. 1). A pale green culture plated out in agar showed green, pale green and colorless colonies of *Euglena*. Isolations from these colonies formed green, pale green or colorless cultures, which have been stable to date (through 8 subcultures over a period of more than 60 days).

The pale green stock cultures persisted for about 3 weeks through 6 or 7 subcultures. The condition disappeared spontaneously; the cultures becoming dark green and of adequate density in 2 or 3 days after inoculation.

We do not know the cause of the variability; perhaps it could have been avoided by maintaining stock cultures in a mineral medium free of avail-

able organic carbon. We concluded that variability of *Euglena* may be an important factor in its use in bioassay for B_{12}.

Standard B_{12} and procedure. We considered the possibility that variations in our standard curves might be the result of the presence in our "pure" B_{12} of B_{12a} which is less stable than B_{12}, or that our procedure in dilution might be at fault. We compared two samples of B_{12}, one of which was crystalline and was obtained through the courtesy of Dr. T. H. Jukes; the other was in solution in ampoules prepared by Eli Lilly and Company and purchased locally.

Dilutions were prepared from three solutions, each containing 0.1 μg. of B_{12} per ml. One of these (A) was made by diluting a solution of crystalline B_{12} containing 1 μg. per ml.; the second (B) was prepared a week later by diluting a solution of the same material at 10 μg. per ml.; the third (C) was prepared from the Lilly material containing 15 μg. per ml. The 3 samples gave essentially the same results (table 1).

TABLE 1. *Optical density in units on Klett-Summerson photoelectric colorimeter of 3 preparations of vitamin B_{12}. Samples A and B prepared from B_{12} from Lederle Company; sample C from B_{12} from Eli Lilly Company.*

$\mu\mu$g. B_{12} per ml.	Net Optical Density		
	A	B	C
1.000	18	16	16
3.125	48	49	57
6.25	102	105	107
12.5	217	219	229
25.0	371	387	391
50.0	491	503	497
100.0	535	519	559
200.0	567	579	615

Results. A variety of natural products have been examined for vitamin B_{12} with the following results:

Cotton. Five g. of dry cotton were thoroughly wet with 50 ml. of boiling distilled water. The water was pressed out of the cotton by means of a glass rod, about 25 ml. being recovered. Extracts of four types of cotton were assayed for B_{12}; cotton obtained through the courtesy of Dr. J. R. Jackson in the field from cotton bolls, two samples of ordinary commercial cotton, a sample of non-absorbent cotton and one of an absorbent cotton. There was some evidence of inhibitory material in these extracts when used at 2× dilution, greater dilutions (4×, 10× or 20×) gave consistent results as follows: boll cotton, about 70 $\mu\mu$g. per ml.; commercial cotton, 150 $\mu\mu$g. per ml.; absorbent and non-absorbent cotton, from 0 to 1 $\mu\mu$g. per ml.

Assuming 1 ml. of the extract to be equivalent to 0.1 g. of dry cotton the figures given above would represent 0.1 of the B_{12} per g. of dry cotton.

More thorough extraction of the cotton would probably have given higher results.

The activity of cotton extract was destroyed by heating with 0.1N NaOH for 30 minutes. B_{12} added to alkali-treated cotton extract was recovered showing that the lack of growth in the extracts treated with NaOH was the result of the destruction of the B_{12} and not because inhibitory material was formed.

Bone marrow. Yellow bone marrow from beef was heated to boiling in distilled water, filtered and made up so that 1 ml. was equivalent to 0.2 g. of the original material. The extract was somewhat inhibitory at 2× dilution but not at 10×. The bone marrow was found to contain 0.002 μg. B_{12} per g. An extract prepared by boiling the marrow for 30 minutes in distilled water gave comparable results. Determinations in liquids and by the FPD method gave satisfactory agreement.

Shrimps. Three lots of shrimps purchased in the market were examined. Each was ground in a Waring blender with distilled water, boiled briefly and centrifuged. The supernatant liquid was prepared so that 1 ml. was equivalent to 0.1, 0.2 or 0.33 g. of fresh weight. The extracts were used in the assays at dilutions which gave the equivalent of from 0.0001 to 0.01 g. of fresh shrimp per ml. No evidence for material inhibitory for *Euglena* was observed.

The quantity of B_{12} in the three samples ranged from 0.0055 μg. to 0.009 μg. per g. fresh weight or from 0.07 to 0.11 μg. per shrimp. About 80 per cent of the activity was eliminated by boiling 30 minutes with 0.1N NaOH. The activity was not materially affected by heating extracts for 30 minutes at 95° C., by heating extracts with 0.1N HCl, or by using 0.1N HCl instead of distilled water for preparing the original extracts.

Oysters. Extracts of two lots of Cape Cod oysters taken from the shell and one lot of Sealshipt blue point oysters were prepared as were those of shrimps. The extracts contained the equivalent of 0.2 or 0.33 g. of fresh oyster per ml. Solutions containing the equivalent of from 0.000016 to 0.0002 g. of fresh oyster per ml. were assayed. Some inhibition of *Euglena* was observed for solutions containing the equivalent of 0.0001 or 0.0002 g. of oyster per ml.

The quantity of B_{12} ranged from 0.20 to 0.460 μg. per g. or from 3.0 to more than 5.0 μg. of B_{12} per oyster. The B_{12} activity was eliminated by heating 30 minutes with 0.1N NaOH. Heating oyster extracts with 0.1N HCl increased the activity 30 to 50 per cent in some instances. Using 0.1N HCl instead of distilled water in preparing the original extracts had little or no effect. Heating dilute extracts to 95° C. for 30 minutes gave somewhat lower values than pasteurization.

Oyster juice obtained when the oysters were opened contained about 0.01 μg. B_{12} per ml.

Clams. Extracts of three lots of chowder clams (*Mercenaria mercenaria* L.) and one lot of little neck clams (*Mya arenaria* L.) were prepared as described for shrimps.

The amount of B_{12} in clam extract as determined by the FPD method was from 10 to 20 per cent of that obtained by optical density in the liquid medium. The values in liquid media for clam extract which had been heated with 0.1N HCl were two or more times those obtained for the untreated extract. Little difference was observed for clam extract heated briefly to 95° C. with 0.1N HCl and that heated for 30 minutes. For example, the average of 6 determinations on one clam extract with the FPD method was 0.0054 μg. per g. of fresh clam; in liquid the average of 6 determinations was 0.0446 μg. per g.; the average of 3 determinations on the acid treated extract was 0.180 μg. per g.

TABLE 2. *Assays in liquid for vitamin B_{12} on extracts of four lots of clams. Sample C, little neck clams (Mya arenaria* L.*); Samples A, D and F, chowder clams (Mercenaria mercenaria* L.*). Note that heating with acid increased B_{12} activity 2 to 5 times.*

Extract		μg. B_{12} per g. fresh clam			
		A	C	D	F
Untreated					
	1	0.046	0.057	0.0370	0.0235
	2	0.044	0.069	0.0485	0.0323
	3	0.049	0.086	.....	.....
	Av.	0.046	0.071	0.043	0.028
Acid treated					
	1	0.1575	0.1135	0.1260	0.1390
	2	0.2020	0.1537	0.2000	0.1475
	3	0.1790	0.1475	.....	.....
	Av.	0.1800	0.1380	0.1630	0.1430

A comparison of untreated and acid treated extract for 4 lots of clams is given in table 2. All determinations were made in liquid media. The acid treated clams averaged about 0.15 μg. B_{12} per g.; more thorough extraction would probably have increased this figure somewhat.

We believe the differences noted above are because B_{12} in the clam exists for the most part (perhaps 95 per cent) in a combined form which is relatively indiffusible. Since the FPD method depends upon the diffusion of the B_{12} through agar the results for a slowly diffusing material would be lower than in liquid media where the substance would merely be required to diffuse into the Euglena cell. Heating with 0.1N acid probably frees the B_{12} from its combined form (fig. 2). As a corollary to the above it may be necessary to assume that *Euglena* is able to utilize the combined form when it penetrates the cell.

Clam juice collected when the clams were opened contained from 0.0014

to 0.010 μg. of B_{12} per ml. Treating clam juice with acid had little or no effect on the B_{12} activity.

The activity of a series of dilutions of clam extract and of crystalline B_{12} gave similar curves (fig. 3). Heating clam extract and acid treated clam extract for 30 minutes with 0.1N NaOH almost completely eliminated the activity, but B_{12} added to the alkaline treated material was recovered. Crystalline B_{12} added in a series of amounts to clam extract at 2000× dilution was recovered. Extracting clams with 0.1N HCl instead of water gave values of the same order as those for acid treated clam extract.

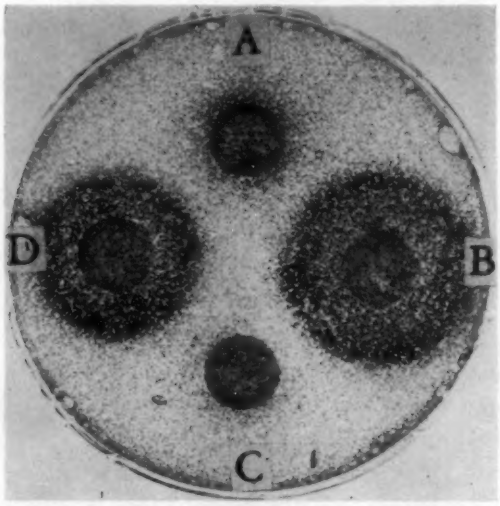

FIG. 2. Filter paper disc tests with clam extracts and same extract heated with 0.1N HCl. A, clam extract equivalent to 0.11 g. fresh clam per ml.: B, same extract treated with 0.1N HCl; C, clam extract equivalent to 0.055 g. fresh clam per ml.; D, same extract treated with 0.1N HCl.

On the basis of our determinations little neck clams contain more than 1.0 μg. of B_{12} per clam; chowder clams from 2.5 to over 3.0 μg. of B_{12} per clam.

A sample of canned minced clams (F. H. Snow Canning Co.) contained between 70 and 80 μg. of B_{12} per 8½ oz. can. This is about 0.380 μg. per g. of fresh clam, a value substantially higher than we obtained for clams removed from the shell and extracted as described earlier. The higher value for the canned clams may be the result of more complete extraction. Acid treatment of the canned clams did not increase activity which suggests that the combined B_{12} was freed in the treatments involved in canning.

Earthworms. We examined several lots of earthworms obtained from a compost pile. The worms were identified through the courtesy of Dr. G. E. Gates as *Pheretima hilgendorfii* (Michaelson). The worms were kept for a day or two in moist leaves and grass to permit them to clean themselves of casts.

Extracts were prepared as for shrimps with 1 ml. equivalent to from 0.5 to 0.9 g. of fresh worms.

Assays of B_{12} in earthworms were affected by the dilution at which the

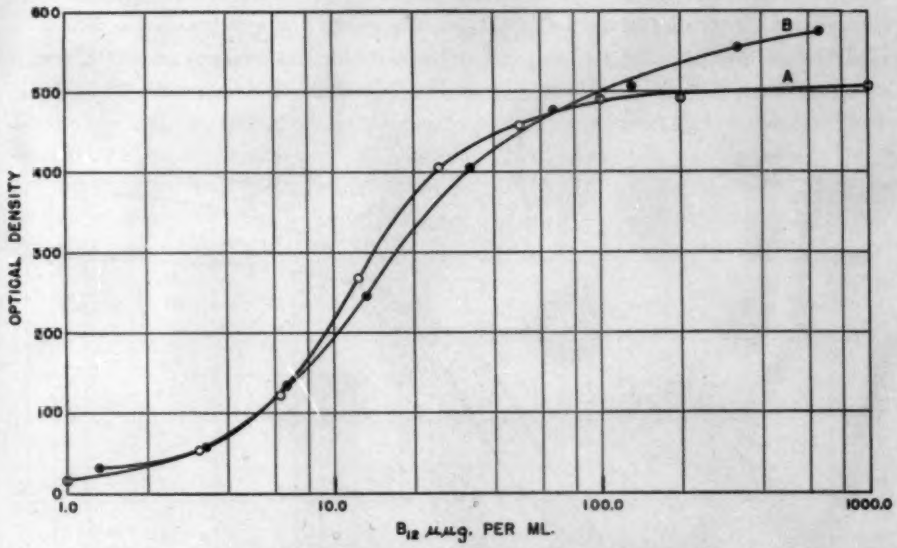

FIG. 3. Optical density of *Euglena* grown in solutions containing crystalline B_{12} and in dilutions of a clam extract. The extract was equivalent to 0.33 g. fresh clam per ml. and contained 0.0133 μg. B_{12} per ml. The dilutions ranged from 20 to 10,000 × original extract. Curve A, pure B_{12}; curve B, clam extract.

extract was heated and the degree to which it was heated. The more concentrated and severely heated dilutions gave the lowest values (table 3). We have not determined whether this is because the earthworm contains a

TABLE 3. *Earthworm extract (F), 1 ml. equivalent to 0.5 g. fresh worm, heated at 3 dilutions to various degrees. All determinations at 5000× dilution of the original extract and expressed as μg. B_{12} per ml. of original extract.*

Dilution Heated	Heat treatment		
	Pasteurized 95° C.	30 minutes at 95° C.	Autoclaved
2x	0.0156	0.0110	0.0120
10x	0.0330	0.0125	0.0080
50x	0.0430	0.0260	0.0125

less stable form of B_{12} or because inhibitory material was formed by the heating.

An earthworm extract (D) containing the equivalent of 0.5 g. worm per ml. was run at a series of dilutions ranging from 100 × to 20,000 ×. Each

dilution was pasteurized at 95° C. The average B_{12} content was 0.092 μg. per g. fresh weight. The curve obtained (fig. 4) exceeded the B_{12} curve at the least dilutions perhaps because the earthworm extract supplied material other than B_{12}, the deficiency of which limited growth in our medium I. In the steeper part of the curves, that for the earthworm extract was lower.

At low dilutions and with pasteurization the B_{12} content of earthworms ranged from 0.08 to 0.180 μg. per g. fresh weight or from 0.21 to 0.54 μg. per average worm.

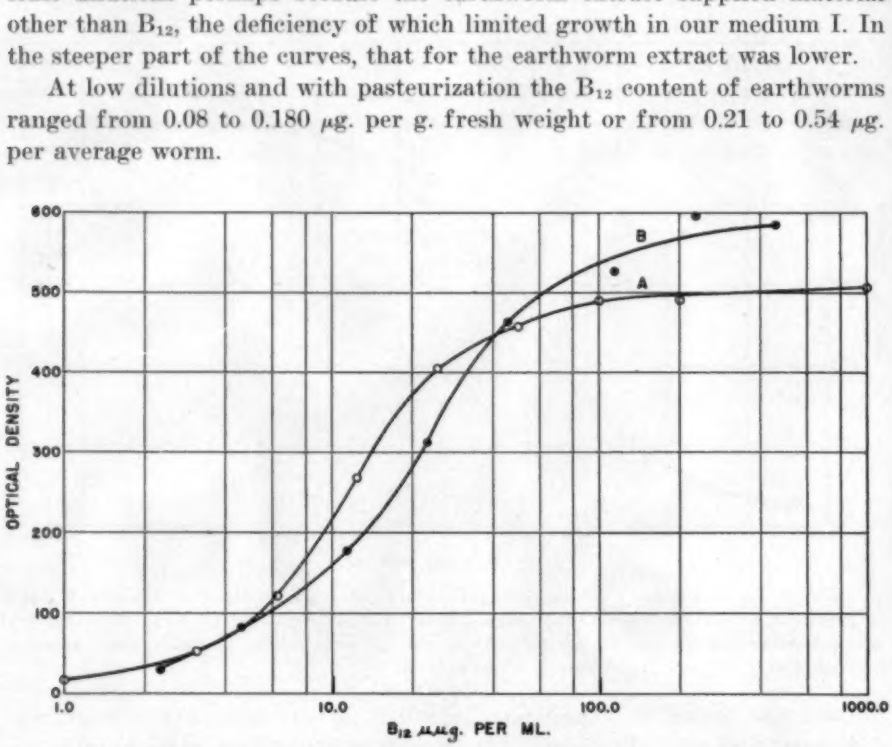

FIG. 4. Optical density of *Euglena* grown 7 days in solutions of crystalline B_{12} and in dilutions of an extract of earthworms. The earthworm extract was equivalent to 0.5 g. fresh earthworm per ml. and contained 0.046 μg. B_{12} per ml. The dilutions ranged from 100 to 20,000 × original extract. Curve A, pure B_{12}; curve B, earthworm extract.

On a dry weight basis the B_{12} per g. of earthworm casts was about 2 per cent of that of the earthworm itself.

Algae. Seventeen species of green, red and brown algae were obtained from Woods Hole, Mass. through the courtesy of Miss Hannah Croasdale. The algae were rough dried on newspapers and then more thoroughly dried in a laboratory drier. Samples as free of epiphytes as possible were collected. They were kept in a dry condition for four years before determinations of B_{12} were made.

A weighed portion of each alga was ground in a mortar with a small amount of distilled water; only partial fragmentation of some of the samples could be accomplished. The boiled samples were centrifuged or squeezed through cheesecloth to obtain extract for analysis.

Assays were made in liquid on the extract of 0.0001 to 0.025 g. of dry alga per ml. depending upon the amount of B_{12} present. For the FPD method the extract of 0.2 g. of alga per ml. was used.

The B_{12} content ranged from less than 0.0001 μg. to 0.1 μg. per g. of dry alga. *Ceramium rubrum* (Huds.) C.Ag., *Champia parvula* (C.Ag.) Harv., *Chondria tenuissima* (Good et Woodw.) C.Ag., *Enteromorpha intestinalis* (L.) Link, and *Lomentaria Baileyana* (Harv.) Farlow contained from 0.05 to 0.1 μg. B_{12} per g. The determinations for *Agardhiella tenera* (J.Ag.) Schmitz, *Chondrus crispus* (L.) Stackh., *Polysiphonia variegata* (C.Ag.) Zanard., and *Ulva Lactuca* L. showed from 0.01 to 0.045 μg. per g. *Ascophyllum nodosum* (L.) LeJol., *Chorda Filum* (L.) Lamour, *Chordaria flagelliformis* (Müll) C.Ag., *Fucus spiralis* L., *Fucus vesiculosus* L., *Laminaria Agardhii* Kjell., *Mesogloia divaricata* (C.Ag.) Kütz, and *Sargassum Filipendula* C.Ag. contained less than 0.01 μg. per g.

TABLE 4. *Vitamin B_{12} content ($\mu\mu$g. B_{12} per g.) of tomato cuttings rooted in Pfeffer's solution. To one set about 1000 $\mu\mu$g. B_{12} per ml. was added to solution 7 days before the first determinations.*

	21 days	31 days	B_{12} added to solution	
			21 days	31 days
Roots	600	780	2400	.....
Basal stem	190	.....	380	.....
Upper stem	80	70	60	70
Older leaves	40	280	95	400
Young leaves	40	340	65	300
Flowers	.....	260	.....	.....
Solution	.....	70 per ml.	.....	640 per ml.

Our determinations probably do not indicate the maximum amount of B_{12} present in these samples. More complete methods of extraction would probably have given higher results.

Ceramium rubrum, Chordaria flagelliformis, Sargassum Filipendula, Laminaria Agardhii and *Ulva Lactuca* were fragmented and boiled in the presence of 0.1N HCl. The acid was neutralized and B_{12} determined in liquid media. The results were equal to or lower than those obtained by extraction with distilled water.

Blue-Green Algae. Through the courtesy of Dr. Folke Skoog and Dr. Gerald C. Gerloff a sample of *Plectonema Nostocorum* Gom. was obtained. This alga had been grown through 28 transfers as a pure culture in an inorganic medium except for a small amount of ferric citrate and citric acid (Gerloff, Fitzgerald and Skoog 1950). Between 0.06 and 0.07 μg. of B_{12} per g. of dry material was found in this sample. This amount is of the same order of magnitude as was found for the more potent marine algae.

Three additional blue-green algae, also furnished by Dr. Gerloff, contained much larger quantities of B_{12}. Extracts of these were prepared containing the equivalent of 2 or 4 mg. dry material per ml. and were assayed at 2, 20, 200 and 2000 × dilutions. Strong inhibition was noted at 2 × dilution. The 20× and lower dilutions gave concordant results. *Calothrix parietina* Born. and Flah. contained 0.64 μg.; *Aphanizomenon flos-aquae* Born. and Flah., 0.285 μg. and *Diplocystis aeruginosa* (Kütz) Trevis 0.24 μg. B_{12} per g. dry weight. *Calothrix* had passed through 19 transfers in pure culture; *Aphanizomenon*, through 35 and *Diplocystis* through 40. A sample of the uninoculated culture fluid from Gerloff showed no B_{12}.

Tomato cuttings. Cuttings of field grown tomato (var. Rutgers) were allowed to root in a modified Pfeffer's solution; a single cutting was placed in a 500 ml. Erlenmeyer flask with about 250 ml. of solution. After two weeks the old solutions were discarded and the well-rooted cuttings were divided into two lots. One lot was placed in fresh Pfeffer's solution; the other in about 250 ml. of Pfeffer's solution to which 0.001 μg. of B_{12} was added per ml. After 1 week's contact with the fresh solutions cuttings were selected from each lot, washed with distilled water and separated into roots, basal stem, upper stem, older leaves and young leaves. Many of the culture solutions had developed considerable growth of algae.

Each sample was ground in a mortar with a small amount of distilled water, boiled and centrifuged. The supernatant liquid was made up so that 1 ml. was equivalent to 0.25 g. of fresh material. Assays for B_{12} were run in liquid using a 2x and 20x dilution of the extracts. The 2x dilution was somewhat inhibitory. Ten days later additional samples were collected, but the extracts were prepared so that 1 ml. was equivalent to 0.1 g. of fresh material.

The cuttings grown without added B_{12} had more B_{12} per g. of fresh material in the roots than in the other parts of the plant; there was little change between 21 and 31 days (table 4). The leaves contained substantially more B_{12} per g. of fresh weight after 31 days than after 21 days.

The significance of these results from the standpoint of synthesis of B_{12} by the tomato plant is invalidated by the presence of B_{12} in the solution in which the cuttings were grown. This was probably formed by the microorganisms (bacteria and algae) which developed in the solution. Sterile Pfeffer's solution inoculated with a bit of the solution from the flasks in which cuttings had grown, developed a good growth of algae and was found to contain 11 μμg. of B_{12} per ml. after 8 days and from 15.0 to 24.0 μμg. after 10 days.

The roots and basal portion of the stem of cuttings grown in the presence of added B_{12} had a higher content of the vitamin than comparable parts of cuttings grown without added B_{12}. This may have been the result in part of B_{12} on the surface of the roots and basal portion of the stem. The upper

stem and leaves contained amounts of the same order as those of the plants in solutions to which no B_{12} was added, indicating slow absorption and transport of the vitamin.

The roots of field grown tomatoes were found to contain up to 15000 $\mu\mu$g. of B_{12} per g. of fresh weight as compared to 2400 in the roots of the cuttings grown in the presence of added B_{12}.

Decisions on the synthesis of B_{12} by tomato must wait for determinations on plants grown under sterile conditions.

Discussion. The greater sensitivity of *Euglena* as compared to *Lactobacillus leichmannii* makes it a preferred organism for the assay of some types of material. For example, Chow, Lang, Davis, Conley and Ellicott (1950) using *Lactobacillus leichmannii,* report that the urine of normal subjects showed no B_{12} activity. We have had no difficulty in assaying B_{12} in urine from normal subjects using *Euglena* and a 10× or 20× dilution of the urine. Hutner (1936) in his original studies with *Euglena* found stimulating material in urine and Ross (1950) has used *Euglena* for assays of urine.

A comparison on a dry weight basis of the vitamin B_{12} content of the materials reported in this paper shows that oysters contained the largest amounts, from 1.0 to 2.3 μg. per g. Clams, earthworms and the blue-green algae (*Calothrix, Aphanizomenon* and *Diplocystis*) were next richest ranging from 0.24 to 0.9 μg. per g. Shrimps, some of the marine algae and *Plectonema* had from 0.03 to 0.1 μg. per g. Bone marrow contained about 0.0025 μg. and cotton (boll and commercial) from 0.00007 to 0.00015 μg. per g.

Zucker and Zucker (1948) found oysters to be about as rich in animal protein factor as liver powder for four week old rats raised on a deficient diet; earthworms also were active but substantially less so than oysters. These observations agree with our findings on the B_{12} content of oysters and earthworms since the former were substantially richer in B_{12} than the latter. Tappan, Lewis, Register and Elvehjem (1950) found 3.0 μg. B_{12} per 100 g. of fresh oysters by rat growth assay. This is much less than our results by Euglena assay. The B_{12} present in oysters, clams, earthworms and shrimps is probably derived and stored from their food though the possibility that they synthesize the vitamin is not eliminated by our observations.

The condition of vitamin B_{12} in the clam deserves further attention. Wijmenga, Veer and Lens (1950) found no free B_{12} in liver. They concluded that it existed in a combined form, probably with a protein or peptide, which might be split by HCN or by proteolysis. Our results suggest that B_{12} exists in the clam in a combined form which is split by 0.1N HCl. In this respect the clam was unique for the materials we examined. With the exception of a minor effect of acid on oyster extract, no such action of dilute acid was observed for various marine algae, for shrimps, oyster juice,

clam juice, earthworms or for tomato roots, stems and leaves. Is the combined form of B_{12} in clams the same as that in liver? Is the B_{12} set free from clams by the normal process of digestion?

Of particular interest is the presence of B_{12} in plant products. We did not expect to find up to 0.6 μg. of B_{12} per g. dry weight in the blue-green algae or as much as 0.1 μg. of B_{12} per g. in the marine alga, *Lomentaria Baileyana*, and nearly as much in *Ceramium rubrum*. Nor did we expect to find B_{12} in cotton taken from the cotton boll. Since the marine algae examined were obtained from natural sources and the cotton was not necessarily from unopened bolls, no conclusions can be drawn on whether marine algae or the cotton plant synthesize B_{12}. In both instances the B_{12} may have been derived from microorganisms associated with the algae or present in the open cotton boll. The same comments may be made for the B_{12} in tomato cuttings.

The blue-green algae, however, have the ability to synthesize B_{12} since our determinations were made on samples of the organisms grown under sterile conditions in synthetic solutions lacking B_{12}. On this evidence the blue-green algae may be considered to be one of the primary sources of B_{12} for water-inhabiting creatures which require the vitamin but lack the ability to synthesize it.

The pressure away from meat and toward vegetarian diets suggests further search for plants which are relatively rich in vitamin B_{12} and the determination of their effectiveness as food supplements.

SUMMARY

1. Vitamin B_{12} activity for a number of natural products was determined by the growth of *Euglena gracilis var. bacillaris*.

2. Variability of *Euglena* and the presence of inhibitory substances in extracts of natural materials influenced the results of assays.

3. Ordinary commercial cotton contained 1500 $\mu\mu$g. B_{12} per g.; cotton from cotton bolls, 700 $\mu\mu$g. and absorbent or non-absorbent, 10 $\mu\mu$g. or less. Yellow bone marrow contained 0.002 μg. per g.; shrimps per g. fresh weight, from 0.0055 to 0.009 μg.; oysters from 0.2 to 0.460 μg.; clams, from 0.14 to 0.18 μg.; earthworms, from 0.08 to 0.180 μg.; marine algae from less than 0.0001 to 0.1 μg. per g. dry weight. Oyster juice contained per ml. 0.01 μg. and clam juice 0.0014 to 0.010 μg. On a dry weight basis earthworm casts contained about 2 per cent as much B_{12} as the earthworm. The B_{12} activity of an extract made from fresh clams was increased two or more times by heating with 0.1N HCl.

4. Blue-green algae grown under sterile conditions in a medium free of B_{12} contained up to 0.64 μg. per g. dry weight. Blue-green algae have the

ability to synthesize substances with vitamin B_{12} activity and are considered to be one of the primary sources of this vitamin for water animals.

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AND
THE NEW YORK BOTANICAL GARDEN

CORRECTION:

In our earlier paper in the Bull. Torrey Bot. Club 77: 429, the vitamin B_{12} for 7,000,000 organisms as found by Hutner et al should read $1.5 \times 10^{-4}\mu g.$ B_{12} per ml. and our figure for 5,000,000 organisms should read $1.5 \times 10^{-4}\mu g.$ B_{12} per ml.

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THE GENUS DELPHINIUM IN NORTH AMERICA: SUPPLEMENTARY NOTES AND DISTRIBUTION RECORDS

JOSEPH EWAN

This series of notes supplements my "Synopsis of the North American species of *Delphinium*" (Univ. Colo. Stud. D, **2**: 55–244. *58 figs.* 1945). The abbreviations for herbaria of deposit used in this paper likewise follow the Index Herbariorum (Chron. Bot. **5**: 142–150. 1939; **6**: 377–378. 1941). The following notes are arranged alphabetically by species.

1. DELPHINIUM ALPESTRE Rydb. Bull. Torrey Club **29**: 146. 1902. This rare endemic *Delphinium* of Colorado was doubtfully admitted as growing on West Spanish Peak, Huerfano Co., on the basis of an immature specimen taken there by Rydberg and Vreeland (no. 6218), on July 9, 1900. Though a close lookout was kept for this larkspur on the peak on my ascent on July 28, 1949 no plants were detected, but typical plants were found growing up through the stems of low twiggy brush about rock outcrops on the open tundra slope of nearby Trinchera Peak, of the main Culebra Range to the west, at 12,150 feet (*Ewan 18,129,* NO). As is usual at the other localities where this species has been found, this tundra *Delphinium* was not common on Trinchera Peak. A form with larger leaf blades and few-flowered racemes was found in association with the more typical plants at the same elevations (*Ewan 18,130,* NO). The follicles are still unknown.

2. DELPHINIUM AMERICANUM Mill. Gard. Dict. ed. 8. no. 7. 1768; evidently based on garden grown plant which Philip Miller says "grows naturally in America."

Although the application of this name must await an examination of collections preserved at the British Museum (Natural History), it is entirely possible that "*Delphinium americanum* Mill." is the correct epithet for the larkspur now known as *D. exaltatum* Ait. The few pertinent facts bearing on this problem are these: Philip Miller adopted Linnean binomial nomenclature in the eighth edition of his *Gardener's Dictionary* (1768) and this work included many new names. Though Miller, when publishing *Delphinium americanum,* did not refer to the figure he published eight years previously in his *Figures* (p. 167, *pl. 250. f. 2.* 1760 [plate dated Aug. 25, 1758]) under a polynomial, it seems likely that the same American source is involved in both publications. Therefore, since we knew from Aiton's publication of *Delphinium exaltatum* that the source of the seeds grown by Philip Miller in 1758 at Chelsea Gardens was Mr. John Bartram (cf. my "Synopsis," l. c. 143. 1945), it is certainly reason-

able to conclude that the basis of Miller's own name, *D. americanum*, was John Bartram's seed collection made somewhere in the Atlantic States. Another possible source may be the basis of this name, however, and an examination of the herbarium of Philip Miller, purchased by Sir Joseph Banks and preserved in the British Museum, should disclose which interpretation is the correct one.[1] The alternate origin of Miller's garden grown plants is seed communicated by Thomas Dale. Miller refers to "Thomas Dale's Observations on many new plants which he discovered in America. MS." Biographical facts regarding Thomas Dale are both scant and dubious but Britten and Boulger refer (Biog. Ind. Brit. & Irish Bot. ed. 2. 82. 1931) to his having probably been a nephew of the apothecary and physician, Samuel Dale (1659–1739), and himself a graduate of Leyden (M.D., 1723). Thomas Dale was a resident of Charleston, South Carolina, from about 1730 and sent plants to Samuel Dale which are now in the British Museum. Emma B. Richardson has called attention to the overlooked source of information about this period in the early Carolina newspapers (Charleston Mus. Leafl. **15**: 11. 1941). Charleston's first newspaper, the *South Carolina Gazette*, dates from 1731–32, and a search through its columns may disclose some mention of Thomas Dale and his botanical activities.

Philip Miller enumerates both *Hortus Cliffortianus* (1736) of Linnaeus and *Flora Virginica* (1739) of Gronovius as his sources in the eighth edition of his *Gardener's Dictionary* but neither contain any references bearing on *Delphinium americanum*. Neither Aiton (Hort. Kew. ed. 2. **3**: 320. 1811) nor de Candolle (Syst. **1**: 357. 1818) mention Miller's species in the respective accounts of the genus.

Both Huth, monographer of the genus *Delphinium*, and the *Index Kewensis* refer *D. americanum* Mill. dubiously to "D azureum?," that is, *D. carolinianum* Walt. Indeed, Philip Miller's plate in his *Figures* (1760), referred to above, is confusing insofar as the foliage clearly illustrates *D. exaltatum* whereas the flowers more nearly represent *D. carolinianum!*

3. DELPHINIUM BAKERI Ewan, Bull. Torrey Club **69**: 144. 1942. The second collection for this remarkable Californian species is: Tomales, Marin Co., April 30, 1923, *Gertrude Raven s. n.* (CAS).

4. DELPHINIUM CHAMISSONIS Pritz. ex Walpers, Repert. **2**: 744. 1843. A record for the interior of Alaska for this species is: Oolamnagavik River, north of Brooks Range, west of Killik River, June 1946, *R. M. Chapman 57* (US).

5. DELPHINIUM × CONFERTIFLORUM Wooton, Bull. Torrey Club **37**: 33. 1910. Two additional collections of this larkspur provide additional evi-

[1] Other plants from Philip Miller are in Herb. Sloane. John Bartram's own collections from Georgia and Carolina taken in 1765–66 are in Herb. Banks.

dence for the hybrid nature of this plant, as previously suggested op. cit. 207. 1945). Both of these collections match the two previously reported and repeat the combination of characters between *D. virescens* subsp. *wootoni* and *scaposum:*

New Mexico: Yuni Mts., Oct. 19, 1928, *Eastwood 15,711* (CAS)

Arizona: grassy openings in spruce-poplar forest, ele. 8,800 ft., Big Lake, Apache Co., Sept. 3, 1948, *F. W. Gould & M. E. Robinson 5123* (NO).

6. D. DECORUM subsp. TRACYI Ewan. op cit. 100. 1945. *D. antoninum* Eastw. Leafl. West. Bot. **3**: 126. 1942; based on *Eastwood & Howell 9861* from Anthony Peak between Mendocino and Tehama counties, 10 July 1941. Type (CAS 292,754) examined.

The publication of *D. antoninum* Eastw. was overlooked when the account of this supspecies was in preparation, otherwise I would have used the specific name in new combination. Milo S. Baker collected a good series of this subspecies on Anthony Peak on July 7, 1942, both near the Lookout Station (10,253a, b, c, NO) and on the east side of Lookout Ridge (10,254a, b, NO). These topotypes are so variable and display such confusing combinations of characters that the affinities of this larkspur are in doubt. When growing in shifting gravels the plants develop long fibrous root systems, comparable to those ecads of *D. bicolor* in the Montana Rockies (op. cit. 123, 1945). In the Baker collections studied the basal leaves are uniformly long-petiolate, and the backs of the sepals are often densely white-villous. A larkspur growing at Dorleska, Salmon Mts., Trinity Co., 6500 ft., *H. M. Hall 8596* (CAS) is to be referred to this subspecies, as well as a collection from Mt. Eddy, Siskiyou Co., *Eastwood 2001* (CAS).

7. DELPHINIUM DIVERSIFOLIUM subsp. HARNEYENSE Ewan, op. cit. 115. 1945. This subspecies may be reported from Utah: near Black Mt., 6500 ft., Raft River Mts., Boxelder Co., "in moist meadow surrounded by sage [brush]," June 23, 1947, *S. J. Preece Jr. 587* (UT, NO).

8. DELPHINIUM GLAREOSUM subsp. CAPRORUM (Ewan) Ewan, op. cit. 125. 1945. Dr. William H. Baker collected a good series of this subspecies during 1948 on Fairview Mt., Calapooya Range, Lane Co., Oregon, near the southern limit of its range (5553, NO, etc.). It occurs at elevations of from 5500 to 5850 feet on this mountain and, as is frequent among Delphinium species near the limits of their ranges, shows wide morphologic variation at this locality.

9. DELPHINIUM GLAUCUM Wats. Bot. Calif. **2**: 427. 1880. A record of the occurrence of this handsome tall larkspur of the mountain cienegas of the Sierra Navada of California in the San Gabriel Mts. of southern California was unintentionally omitted from the "Synopsis" (135, 1945). The first record was made by Ada Nesta Dunn (Mrs. Joseph Ewan) at Guffys Spring, 8400 ft., Blue Ridge, near San Bernardino-Los Angeles county

line, San Gabriel Mts., on August 12, 1935 (LA, NO) and I recollected the species there the following year, *Ewan 10,013* (NO). A colony of robust plants standing 1–2 m. high grew among the stools of *Carex* and *Juncus* in a small hillside cienega about a natural spring. The larkspur has not been observed elsewhere in the San Gabriel Mountains.

10. DELPHINIUM HUTCHINSONAE Ewan, sp. nov. Stout, fistulous perennial from a short thick rhizomatous rootstock, the stems erect, subsucculent, simple, 45–60 cm. tall, only sparingly leafy above, cano-pubescent to thinly setulose and purplish at the base, the middle and upper portions of the stem glabrous to hirsutulose; leaves green or withering at anthesis, palmatisect into 5 broadly cuneate-obovate primary segments, each division trifid, mucronate with a white callosity and often ciliate distally with pustulate-based hairs, the mid-cauline leaves pentagonal in outline, 2.5–3.5 cm. long, 5–6 cm. wide, impressed-venose and glabrous above, thinly cano-hirsutulose beneath, long-petiolate, the petioles ascending, carinate-clasping at the base, 14–18 cm. long, thinly hirsutulose with white hyaline hairs 0.5–1.0 mm. long; racemes stiffly ascending, the tips of the branches often flaccid and pedicels weak or recurved, more or less pubescent below the flowers, the bracts few, linear-filiform, about 1 cm. long, rather prominent; flowers rather large, showy, dark purple or fading in herbarium specimens, the sepals broadly oval, obovate or suborbicular, barely acute, 15–20 mm. long, 12–16 mm. wide, thinly pubescent over the back, the spur often stoutly decurved or uncinate, 10–12 mm. long; limb of lower petal oval, 6 mm. long, the sinus closed, 2.5 mm. deep, the claw lightly hairy, basal appendage evident; upper petals rhomboid, subentire or unequally notched, venulose; stamens glabrous, dilated at the base, hyaline-membranous; follicles narrowly oblong, small, short, 9–10 mm. long, dark purple-venulose, appressed-puberulent, cusp thin, erect, 2 mm. long; seeds quadrate, dark brown, smooth, 1 mm. long, with a narrow white wing on angles.

Herba perennis caulibus strictis fistulosis, subsucculentis, 45–60 cm. altis, e radice crassa lignosa, simplicibus, ad basim cano-pubescentibus vel setulosis, ad apicem glabris vel hirsutulosis; foliis imprimis ad caulis basim subconfertis, palmatisectis, segmentis cuneato-obovatis divisis trifidis mucronulatis et saepe pustulato-ciliatis ad apicem, 2.5–3.5 cm. longis, 5–6 cm. latis, extus venosis atque glabris, sparse cano-hirsutulosis partis inferioribus, manifeste petiolatis, petiolis dilatatis ad basim, 14–18 cm. longis, sparse hirsutulosis; racemis ascendentibus, ramis superioribus deinde flaccidis; floribus pedicellis saepe recurvatis, insignis, atro-purpureis, sepalis ovalibus, subacutis, 15–20 mm. longis, 12–16 mm. latis, extus sparse pubescentibus, calcar uncinatis, 10–12 mm. longis; petalis inferioribus ovalibus, bifidis, sinus 2.5 mm. profundis appendicis ad basim manifeste; petalis superioribus rhomboideis, subinteger vel subemarginatis; staminibus glabris; folliculis oblongibus, parvis, 9–10 mm. longis, atro-venulosis, puberulentis, seminibus quadratis, 1 mm. longis, glabris, brunneis, angulatis similiter angustis alatis.

TYPE from sand of beach 2½ miles south of Monterey, Monterey Co., California, May 28, 1938, *Susan W. Hutchinson 7423* (COLO; isotype, NO).

It is likely that Bentham was referring to *Delphinium hutchinsonae* when he described as "D. decorum . . . ? forma elatior ramosa, etc." a collection from "sylvis prope Monterey."[2] The relationships of this maritime endemic lie between true *D. decorum,* which is rare at Monterey (cf. Synopsis 99, 1945), and *D. variegatum* subsp. *apiculatum.* The leaves at times suggest *D. decorum* (e.g., sand hills, Pt. Pinos, near lighthouse, May, 1911, *Fidella G. Woodcock s.n.*—NO) but in the type collection are more suggestive of forms of *D. variegatum.* The woody fibrous rootstock, flowers and seeds also most closely resemble the polymorphic *D. variegatum.* The petioles of *D. hutchinsonae,* however, are not pilose as in *D. variegatum.* The leaves have the unusual combination of long petioles and proportionately small blades of the Sierran *D. polycladon* but there is no real affinity with that species. In my key to the California species (op. cit. 83–88, 1945) *D. hutchinsonae* comes nearest *D. blochmanae* for its sepal size, pubescence, and habit, but the leaves, uncinate spur, and interrupted raceme are very unlike that San Luis Obispo County endemic. Of all its characters *D. hutchinsonae* is distinctive for its short, heavy, decurved spur and the short, broad, obovate and blunt sepals.

There is evidently some considerable variation in the size of the flowers, shown by the type collection, and also by a collection from Carmel Bay, Monterey Co., June, 1903, *Elmer 4382* (CAS 89,234). This variation in size may be due to differences in water supply in various habitats, *Elmer 4382* representing an example from a drier site, *Hutchinson 7423,* from a more moist habitat.

A collection made on the Pine Ridge trail, 500 ft., Pfeiffer State Park, above Big Sur, Monterey Co., "mostly under poison oak," May 29, 1937, *Mildred E. Mathias 1301* (NO, LA, COLO, MO) is peculiar for its smaller flowers, more bracteate racemes, and seeds with broader white wing-like angles, and including some individuals with characters of *D. patens* or hybrids with that species. Its identity must await more field work in the Big Sur region correlated with collections made along the sand hills of the Monterey peninsula.

This distinctive *Delphinium* is named for my friend of many years, Susan Whipple Hutchinson (Mrs. William Wilson Hutchnson) of Glendale, California, whose well prepared plant collections have enriched our herbaria (cf. Am. Midl. Nat. **17**: 431). In 1943 she presented her herbarium of plants of western North America to the University of Colorado.

11. DELPHINIUM NUTTALLI Gray, Bot. Gaz. **12**: 51, 54. 1887. Another station for this little known and localized larkspur is: Oregon: Grand Ronde, Polk Co., *Eastwood & Howell 3563* (CAS).

12. DELPHINIUM RAMOSUM forma SIDALCEOIDES Ewan, forma nov. Habit

[2] Benth. Pl. Hartweg. 295. 1848.

rather more strict than *D. ramosum* subsp. *ramosum;* herbage conspicuously glandular-pubescent throughout, even stellate-pubescent on lower or median portions of stem; leaves smaller, pentagonal in outline, 4–5 cm. wide, deeply palmatisect to the base into multifid divisions, each division finely cut into linear-filiform ultimate segments; petioles strictly ascending, appressed against the stem; spur very slender, 10–12 mm. long; follicles unknown.

A *D. ramosum* subsp. *ramosum* caulibus et foliis glandulo-pubescentibus, deinde caulibus regionibus inferioribus minute stellato-pubescentibus; foliis parvioribus, 4–5 cm. latis, palmatisectis ad basim, segmentis linearo-filiformis; racemis congestis; calcar tenuis, 10–12 mm. longis differt.

TYPE from volcanic ash deposits, along the road to Stone Cellar Ranger Station and Saguache Park, near junction of main highway, 4 miles west of Cochetopa Pass, 9700 ft., Saguache Co., Colorado, July 28, 1950, *W. A. Weber 5798* (COLO; isotype NO).

Delphinium ramosum characteristically favors the dappled shade of aspen woodlands or the brushy borders of open meadows, and, therefore, this collection from volcanic ash deposits in the open is exceptional ecologically. The occasional stellate-pubescence of the stems is unique among North American Delphiniums, and its suggestion of the genus *Sidalcea* (Malvaceae) is noticed in the name of this form. Here reciprocal transplant experiments would be of the greatest interest to determine the genetic stability of these morphologic differences in pubescence and the amount of dissection of the leaves over typical *D. ramosum*.

13. DELPHINIUM UMATILLENSE Ewan, Bull. Torrey Club **69**: 149. 1942. John Henry Christ of Portland, Oregon, has made collections of this larkspur in Idaho, where he finds it closely restricted to the Weiser River watershed: 7 miles n. of Council, Adams Co., "on basaltic hillside, with sagebrush, yellow pine and service berry," *Christ 17,907* (NO); 10 miles nw. of Council, on Hornet Creek road, Adams Co., "on east facing hillside with snowberry, cherry, and yellow pine," *Christ 17,908* (NO); 13 miles nw. of Mann Creek Store, 1 mile above Mann Creek Ranger Station, Washington Co., *Christ 17,945* (NO), raceme less dense than typical and possibly colony of another hybrid combination?

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HETEROPHYLLY AND FOLIAR VENATION IN LACUNARIA.

ADRIANCE S. FOSTER

Previous investigations by the writer on the foliar venation of the Quiinaceae have dealt with *Quiina*, *Touroulia* and *Froesia* (Foster 1950a, 1950b). In the present article, the remaining genus *Lacunaria* will be considered, with particular reference to an intensive study of the morphology and venation of the seedling and adult leaves of *Lacunaria crenata* (Tul.) A. C. Smith.

In his original characterization of *Lacunaria*, Ducke (1925 p. 139) stated that the genus is distinguished from *Quiina* and *Touroulia* by the presence of conspicuous latex-containing cavities in the pericarp of the fruit, by the flat and delicate cotyledons of the embryo, and by an extensive development of endosperm in the seed. To these disingtuishing characters Ducke (1930 p. 167) later added the dioecious nature of the flowers and the 8-13 loculate ovary. Smith (1939) accepted Ducke's new genus and concluded that certain members of the Quiinaceae previously assigned to *Quiina* should be regarded as species of *Lacunaria*. Among several proposed transfers to this genus Smith included Tulasne's (1849 p. 163–164) "*Quiina crenata.*" According to Lanjouw and van Heerdt (1940) the presence of latex-cavities in the pericarp is not restricted to *Lacunaria;* similar structures also occur in several species of *Quiina* which they investigated. Nevertheless, Lanjouw and Van Heerdt conclude that the totality of morphological characters justify the recognition of *Lacunaria* as a distinct genus "though it may be possible that there are transitions." (cf. also Lanjouw and van Heerdt 1941 for a detailed taxonomic description of *Lacunaria crenata*). It is hoped that the present study of foliar venation may provide additional morphological evidence for a more thorough taxonomic comparison between *Lacunaria* and the other members of the Quiinaceae. As presently conceived, the genus *Lacunaria* consists of about 15 species of small or medium sized trees and is restricted to tropical America, reaching its greatest development in the Guianas and the Amazon rain forests of Brazil.

Materials and Method. A preliminary survey of the venation of *L. crenata* (Tul.) A. C. Smith was made during the writer's period of study at the Instituto Agronomico do Norte, Belém, Pará, Brazil.[1] In addition, a

[1] Grateful acknowledgement is made to the Guggenheim Memorial Foundation for the Fellowship during the sojourn in Brazil.

number of seedlings of an apparently undescribed species of *Lacunaria* were collected by the writer in an area of the rain forest near the town of Vigia in northeastern Para. One of these seedlings and the fruit and adult foliage of the parent tree have been deposited in the herbarium of the University of California; in the present paper, this material will be designated simply as "*Lacunaria sp.*" pending the ultimate taxonomic disposition of this entity. Special acknowledgement is due Dr. J. Murça Pires of the Instituto who sent seedlings and adult foliage of *L. crenata* which he collected in the Bosqué Municipal on the outskirts of the city of Belém. The writer is also greatly indebted to the following individuals for herbarium loans and leaf specimens of *Lacunaria:* Dr. Theodor Just, Chicago Museum of Natural History; Dr. A. C. Smith, U. S. National Herbarium; Dr. F. P. Jonker, Botanisch Museum en Herbarium, Utrecht; Dr. J. Murça Pires, Instituto Agronomico do Norte, Belém, Brazil; and Dr. J. G. Kuhlman, Jardim Botânico, Rio de Janeiro, Brazil. When referring to specimens from any of the above herbaria, the standard abbreviations proposed by Lanjouw (1939) are used.

The seedling and adult leaves were cleared in NaOH and stained with safranin by the method outlined by the writer (Foster 1949, pp. 216–217). The stained specimens were mounted in "Clarite" and large slides (7.5 × 5 cm.) were employed for most of the preparations. The photomicrographs, as in previous articles on the Quiinaceae, are the skillful work of Mr. Victor Duran and the drawings represented in Fig. 1–3 were prepared by Mr. Raphael Rodriguez.

Morphology of Leaves. The early leaves of the seedlings of both *Lacunaria sp.* and *L. crenata* are conspicuously pinnatifid and very different in form than the adult leaf (fig. 1–3). In *L. crenata* the leaves of shoots developed near the base of cut or injured trees are likewise pinnately-lobed to varying degrees. Whether heterophylly is widespread throughout the genus, or as in *Quiina,* is restricted to certain species remains to be determined. As far as the writer could discover, the only reference to the heterophylly of *Lacunaria* in the literature consists of some brief comments by P. Sagot which are quoted in Planchon and Triana's (1861 p. 313) memoir and which later were condensed in Sagot's (1880 p. 165–169) catalogue of the plants of French Guiana. With reference to "*Quiina crenata* Tul." (= *Lacunaria crenata* (Tul.) A. C. Smith) Sagot states that "the leaves are highly variable in form, entire or crenate and sometimes so deeply crenate that they become nearly pinnatifid." In his catalogue Sagot observes that the leaves in young specimens, developed from seeds, are pinnately lobed.

Striking examples of pinnatifid juvenile leaves, of the type investigated by the present writer, occur on one of the sheets of Sagot's collection No. 1207 of *Quiina crenata.* A photograph of this sheet was furnished the

FIGS. 1–2. *Lacunaria crenata*. FIG. 1. Seedling with decussate stipulate pinnatifid leaves, collected from the Bosqué Municipal, Belém, Brazil. Natural size. FIG. 2. Terminal portion of a shoot of the pistillate parent tree of this seedling, showing the simple verticillate leaves with interpetiolar stipules and the terminal inflorescence. $\frac{1}{4}$ natural size.

writer through the courtesy of the Muséum National d'Histoire Naturelle de Paris.

In both species of *Lacunaria* examined, the lower leaves of the seedlings are decussately arranged in contrast to the strictly verticillate phyllotaxis of the subsequent foliar organs (figs. 1–2). The seedling leaves develop paired narrow-acuminate stipules; these appendages tend to become concrescent and distinctly interpetiolar at the nodes bearing the adult type of leaf. On the basis of the limited material investigated, the seedling leaves seem highly variable in size and in the number and degree of development of the lateral lobes. In *L. crenata* the pinnatifid leaf type ranged from 2.5–6 cm. in length while in a robust seedling of *L. sp.* (fig. 3) the length varied from 6–11 cm. The size and number of lateral lobes fluctuate not only between successive leaf-pairs but also between the members of the same pair. For example, in the seedling of *L. crenata* shown in figure 1, both members of the lowermost and uppermost pairs of leaves agree in possessing three pairs of lateral lobes; leaves situated at nodes between these pairs however have 4, 5 or 6 lateral lobes and variations occur between members of certain pairs. In another seedling of *L. crenata* with nine pairs of leaves, the variation was even more erratic particularly with reference to differences in the number of lateral lobes between members of a given leaf-pair. The early seedling leaves of *Lacunaria sp.* are also variable in respect to the development of lateral lobes (fig. 3a-c). As in *L. crenāta,* one or both members of a leaf-pair may be strikingly asymetrical as the result of the unequal development or the complete suppression of certain lateral lobes (figs. 3a-c and 4).

The transition from the pinnatifid to the simple type of leaf consists in the progressive reduction in size and ultimate suppression of the lateral lobes (fig. 3). As might be anticipated the degree of lobing of the transitional forms varies widely, not only between members of the same pair but even with respect to the two margins of the same lamina. In general, however, the gradual reduction in development of the lateral lobes proceeds acropetally with respect to the leaf apex and the upper lobes maintain their identity longest (fig. 3d-f). A further aspect of the transition, which will be described in detail in the next section of this paper, involves a progressive increase in the number of secondary veins. This progressive elaboration of the major system of veins is clearly depicted in figure 3.

Venation patterns. The literature dealing with the morphology and taxonomy of *Lacunaria* contains only meagre descriptions of the foliar venation. Tulasne (1849 p. 157) characterized the venation of *Quiina* (which included the entity now termed "*Lacunaria crenata*") as "subtly lineate since the tertiary veins are transversely and very elegantly parallel." Ducke (1925 p. 139–140) made no direct use of foliar venation in his origi-

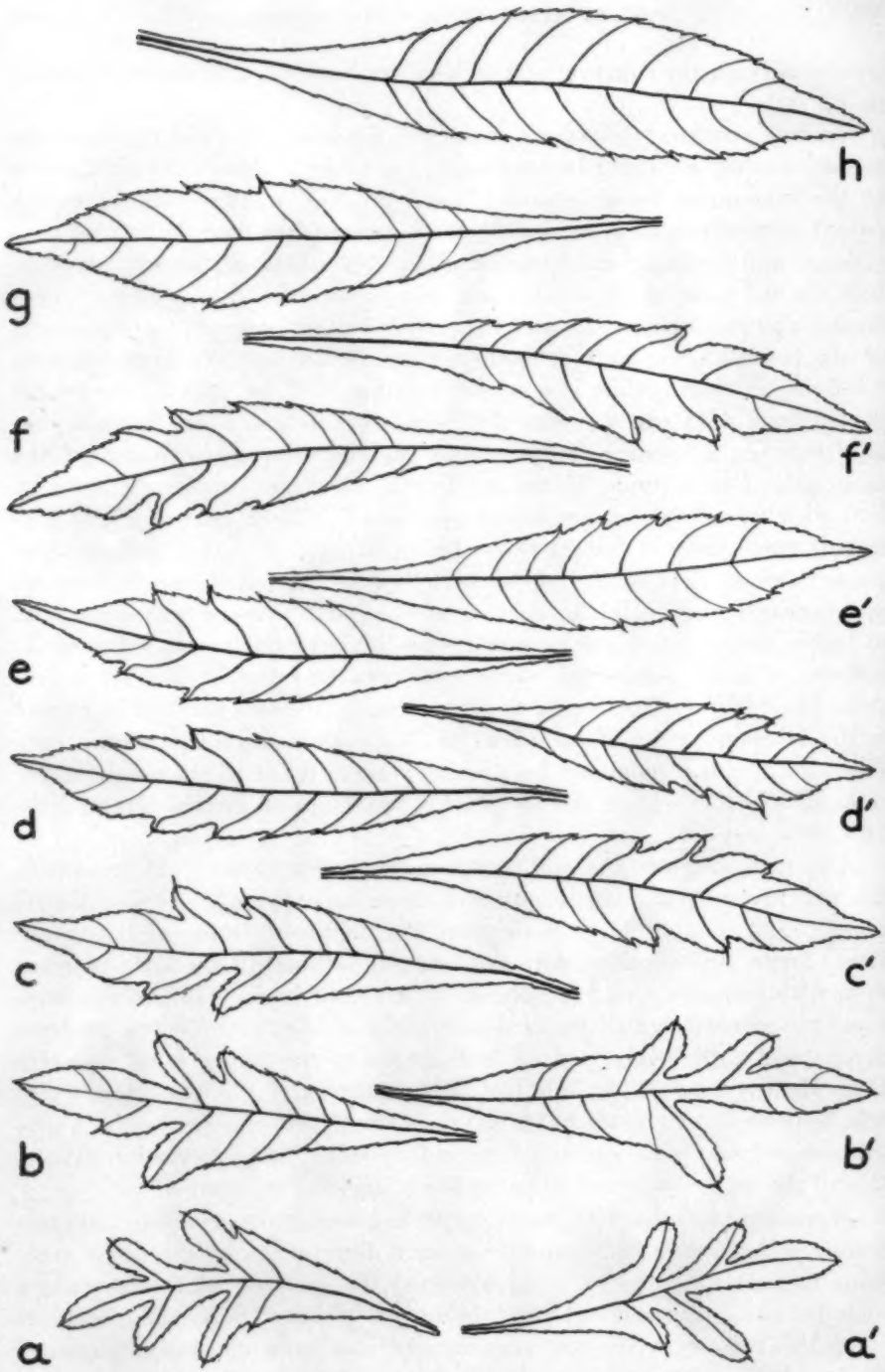


FIG. 3a-h. *Lacunaria sp.* Leaf series from a seedling collected near Vigia, Pará, Brazil, showing the transition from pinnatifid to simple leaves. Only one member of leaf-pairs 3g and 3h is represented. Note progressive increase in number of secondary veins in this series. ¾ natural size.

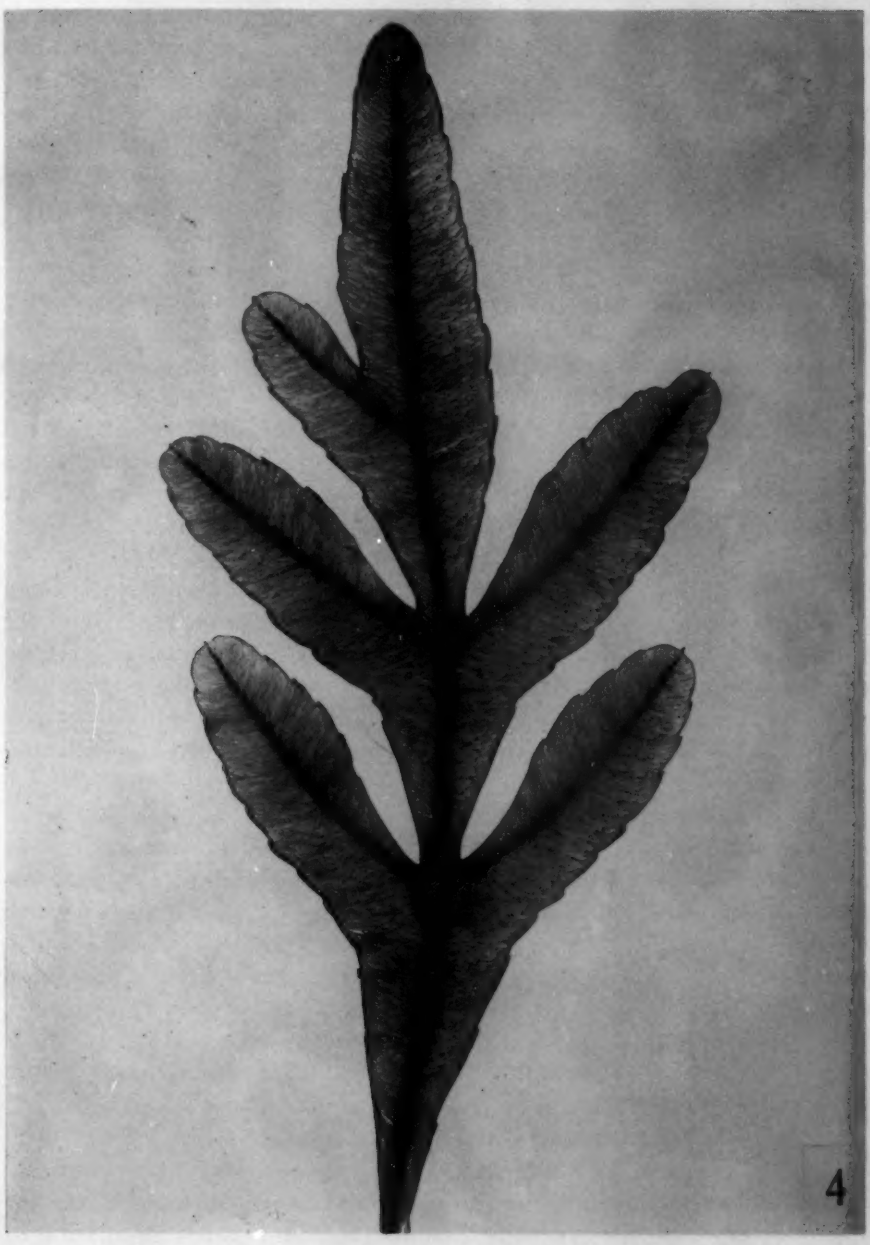


FIG. 4. *Lacunaria crenata*. Deeply pinnatifid seedling leaf showing entire venation pattern. × 11.

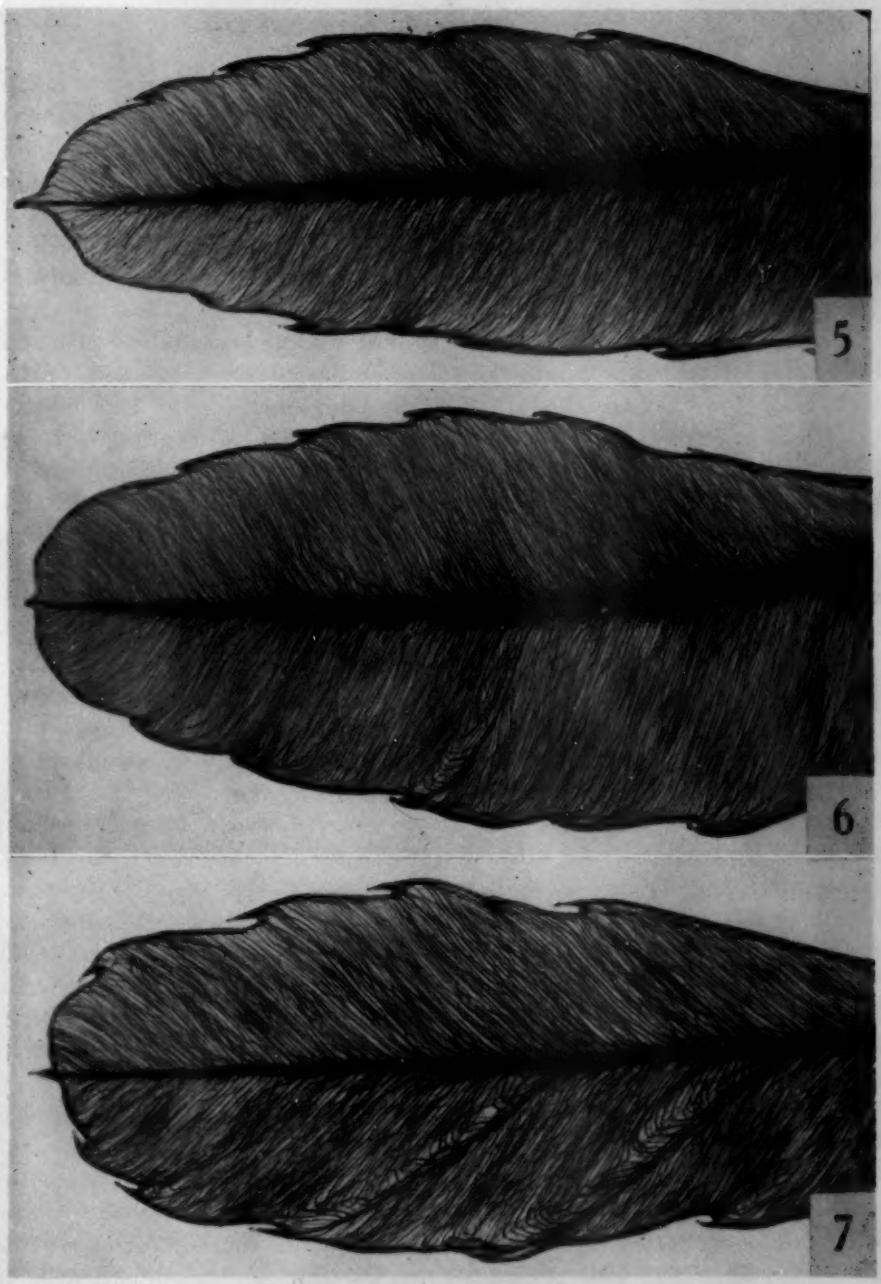
nal diagnosis of *Lacunaria* although he notes under the description of *L. grandifolia* that the leaves "between the ribs below are very densely and very subtly undulate-striate." In his second taxonomic study of *Lacunaria* Ducke (1935 p. 170–171) used the number of secondary veins as a key-character for separating species but made no further reference to the minor venation. Smith (1939) described the gross aspects of the venation of several species noting especially that in *L. coriacea* the "veinlets below are conspicuously striated."

These brief fragmentary descriptions convey a very inadequate picture of the nature of the complex intercostal venation of the leaf of *Lacunaria*. In the two species which were most intensively studied by the present writer, it was possible to compare the venation patterns of seedlings, coppice-shoot, transitional and adult leaf types. Hence, as in the writer's previous studies, the *progressive elaboration* of the venation could be traced and proper consideration given to fluctuations in pattern at successive phases in the ontogeny of the plant as a whole.

Seedling and coppice-shoot leaves. Figure 4 represents the complete venation typical of a lower deeply-pinnatifid seedling leaf of *L. crenata*. The major venation consists of a stout midrib traversing the entire leaf and five conspicuous secondary veins. Each of the these veins extends from the midrib to the tip of the adjacent lateral lobe, dividing the latter into two asymmetrical halves. The minor venation is represented by the very numerous, delicate, closely spaced "veinlets" which diverge from the main vascular system into the terminal and lateral lobes and the wings of the petiole and rachis (figs. 4–5).

When studied at high magnification, the "veinlets" of the minor venation system prove to consist of two distinct categories of strands viz.: (1) true conducting strands composed of spirally-thickened tracheary elements flanked by strands of fibers and (2) fiber-strands or "false veinlets" which are entirely devoid of tracheary elements and consist only of thick-walled fibers (fig. 8). The fiber-strands are very numerous and, either singly or in groups, pursue an undulating course between the true conducting strands. Because of their abundance, their frequent dichotomous branching and their frequent blind termination within the mesophyll, the fiber-strands closely simulate the behavior of the true veinlets.

A careful study of the complete minor venation of several lobes of seedling leaves revealed wide fluctuation in the degree of branching and methods of termination of the true conducting strands. Most commonly the veinlets diverge without branching from the midrib towards the margin of the lobe, ultimately fusing with the tracheids or fibers of the marginal vein or else ending blindly in the mesophyll. In the latter case, the fibers which accompany the tracheary strand diverge near its termination and send in-



FIGS. 5–7. *Lacunaria crenata.* FIG. 5. Lateral lobe of seedling-leaf showing numerous closely-spaced veinlets diverging from the midrib to the margins; tertiary veins are absent. ×11. FIG. 6. Lowermost lateral lobe at left of the seedling-leaf shown in figure 4. Note the tertiary vein in the lower half of this lobe. ×11. FIG. 7. Lateral lobe of a pinnatifid leaf of a coppice-shoot, showing two well-developed tertiary veins in the lower half. Note that an arcuate pattern of veinlets extends the full length of the tertiary veins. ×11.

trusive tips into the mesophyll tissue; a similar behavior is typical of many of the terminations of the false veinlets. A relatively low percentage of the conducting strands are dichotomously branched, the bifurcation taking place either near the attachment of the veinlet to the midrib or more distally (fig. 8). Occasionally one or both shanks of the main dichotomy may fork. When dichotomous branching occurs, one of the two shanks very commonly ends blindly while the other fuses with the marginal vein or with an adjacent fiber-strand.

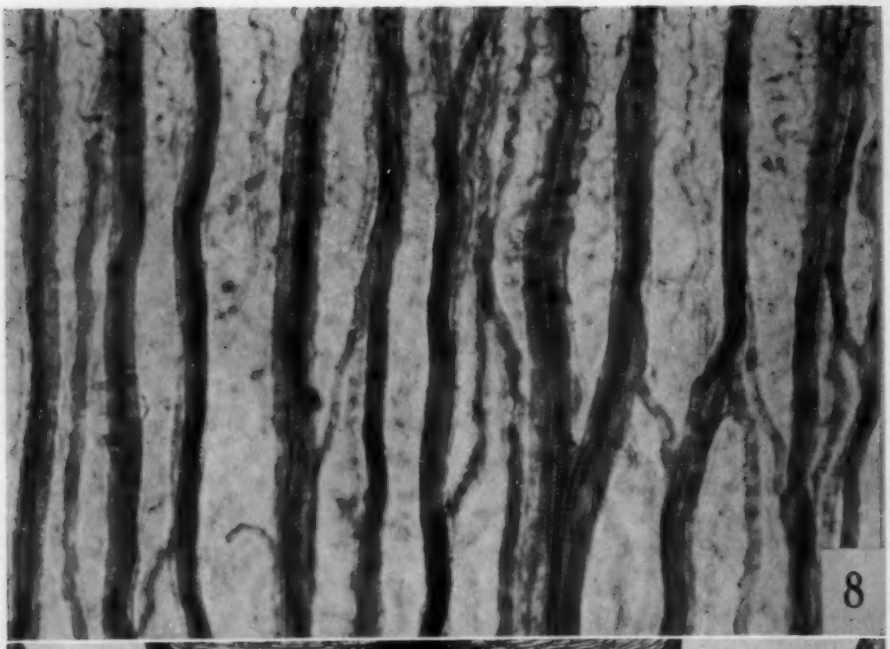
In many seedling leaves of both species, all the strands (fibrous and conducting) which extend into the halves of the lateral lobes are approximately equal in size (fig. 5). But in some seedlings and in all of the coppice-shoot leaves of *L. crenata*, certain of the lateral lobes had developed one or more well defined, lateral veins (figs. 6–7). With respect to the pinnatifid leaf as a whole, these veins should be designated as "tertiary veins" (fig. 4). If however, the lobe itself is used as the point of reference, its lateral veins are "secondary" because they diverge as branches of the primary vein or midrib. To avoid confusion in description, the veins in question will be designated as "tertiary".

From a study of the limited material available, it is impossible to explain or to correlate satisfactorily the apparently erratic pattern of development of the tertiary veins and their absence in the transition and adult leaf types. In some leaves (fig. 4) each lateral lobe had produced one or more tertiary veins; in other leaves of the same seedling, certain lobes, equal in size and appearance to those shown in figure 4 were entirely devoid of tertiary veins. Perhaps most frequently the tertiary vein or veins are restricted to the lower and usually broader half of the lobe of seedling leaves; this also seems to be a definable trend in the leaves of coppice-shoots (figs. 6–7). Rather infrequently, tertiary veins may develop in both halves of the lateral lobe. As is clearly shown in figure 6, a distinctive arcuate pattern of veinlets develops on the acroscopic side of the tertiary veins. In all the seedling leaves examined this arcuate pattern is restricted to the outermost portion of the veins while in the lobes of coppice-shoot leaves the arches extend the full length of the more robust tertiary veins (figs. 6–7). In either case the tertiary vein exhibits a more or less conspicuously plumose character and is consequently quite well-demarcated from the adjacent slender veinlets.

Transition forms. In many of the deeply pinnatifid seedling leaves, the number of secondary veins corresponds exactly with the number of lateral lobes (figs. 3a–a′ and 4). But in other cases, nearly every seedling leaf had developed one or more additional secondary veins in either the terminal lobe or the petiolar wings or in both of these regions. These extra secondary veins, in their relatively delicate structure and erratic pattern of distribution, resemble the tertiary veins of the lateral lobes previously described. The

gradual reduction in prominence of the lateral lobes and the increasing length of the leaf are accompanied by further progressive additions, basally and apically, to the system of lateral secondary veins (fig. 3b–g). In the transitional leaf types shown in figures 3d–f, only four to six of the medianly situated secondary veins are equivalent to the midribs of lateral leaf-lobes; the remainder represent additions to the previous vascular system and terminate in certain of the marginal crenations. The secondary veins of members of the uppermost pairs of leaves (fig. 3g–h) are about three times as numerous as in the lowermost foliar organs of this series, and those corresponding in position to the lobes are not sharply demarcated from their neighbors. Although it was not possible to study in detail the transition from the simple leaves of the seedlings to the similar but larger leaves of well developed trees, it is evident that the number of secondary veins in successively developed leaves must increase for some time. Large laminae of *Lacunaria sp.* may develop 20–22 conspicuous secondary veins at each side of the midrib. The number of secondary veins in large vigorous leaves of *L. crenata* is smaller but yet more numerous than in the leaves of seedlings (figs. 1–2).

The deep sinuses which divide the lateral lobes of seedling leaves from the rachis extend nearly to the point of connection between the secondary veins and the midrib (fig. 4). Concomitant with the reduction of the lateral lobes, the depth of each sinus decreases and there is built up in the tissue between it and the point of divergence of the secondary vein a characteristic arcuate pattern of veinlets (fig. 9). This series of arches represents the confluence between the veinlets derived from the acroscopic side of the secondary vein and those which extend from the adjacent midrib. Above the arches, the veinlets derived from the midrib, as in seedling leaves, extend towards the margins of the rachis-wings. In regions of the lamina of transition forms where lateral lobes are very rudimentary or absent, the intercostal venation closely resembles that typical of the adult type of leaf (fig. 10). Near the axil of the secondary vein, the lateral veinlets are more or less curved in their course but do not collectively form a very conspicuous arcuate pattern. Distal to the midrib, the two secondary veins are interconnected by closely spaced fiber-strands and conducting veinlets. Careful study at high magnification reveals that a much higher percentage of the true vascular strands are dichotomously branched than is the case in the lobes of seedling leaves. In the intercostal area shown in figure 10, the dichotomous branching of the veinlets is particularly evident along the acroscopic side of the lower of the two secondary veins. The shanks of a given dichotomy either end blindly distal to a secondary vein or anastamose in various ways with neighboring strands; some veinlets extend from one secondary vein to the other without branching. As in the lobes of seedling leaves however, there



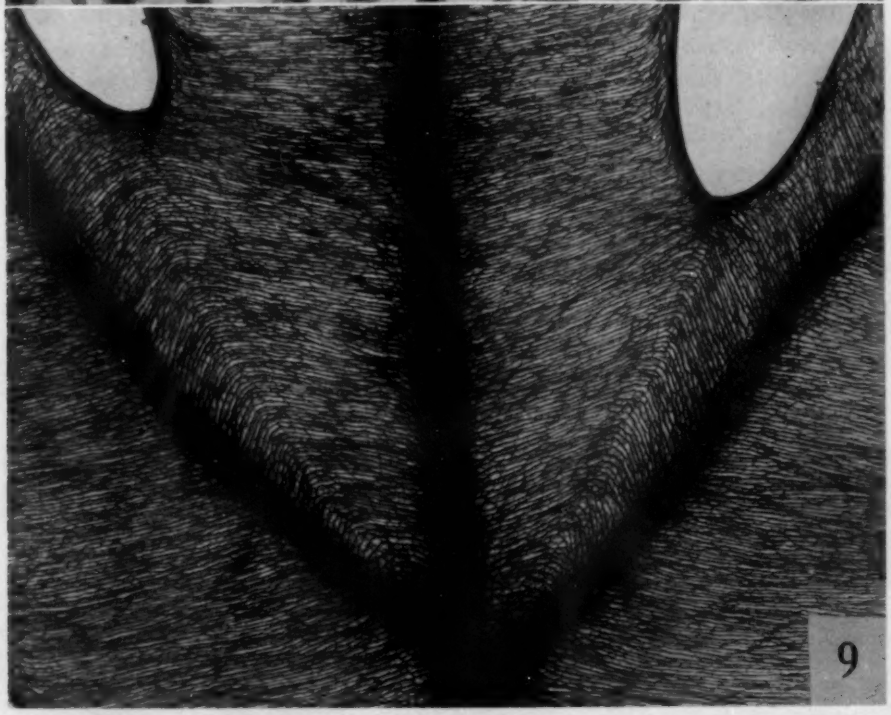


FIG. 8–9. *Lacunaria crenata.* FIG. 8. Details of small portion of the minor venation of the lowest lateral lobe at right of the leaf shown in figure 4. Note the dichotomous branching of one of the tracheary strands and the conspicuous development of fibers and fiber-strands. × 325. FIG. 9. Portion of a transition-form showing the typical arcuate venation pattern which extends from the axil of each of the secondary veins to the border of the sinus. × 11.

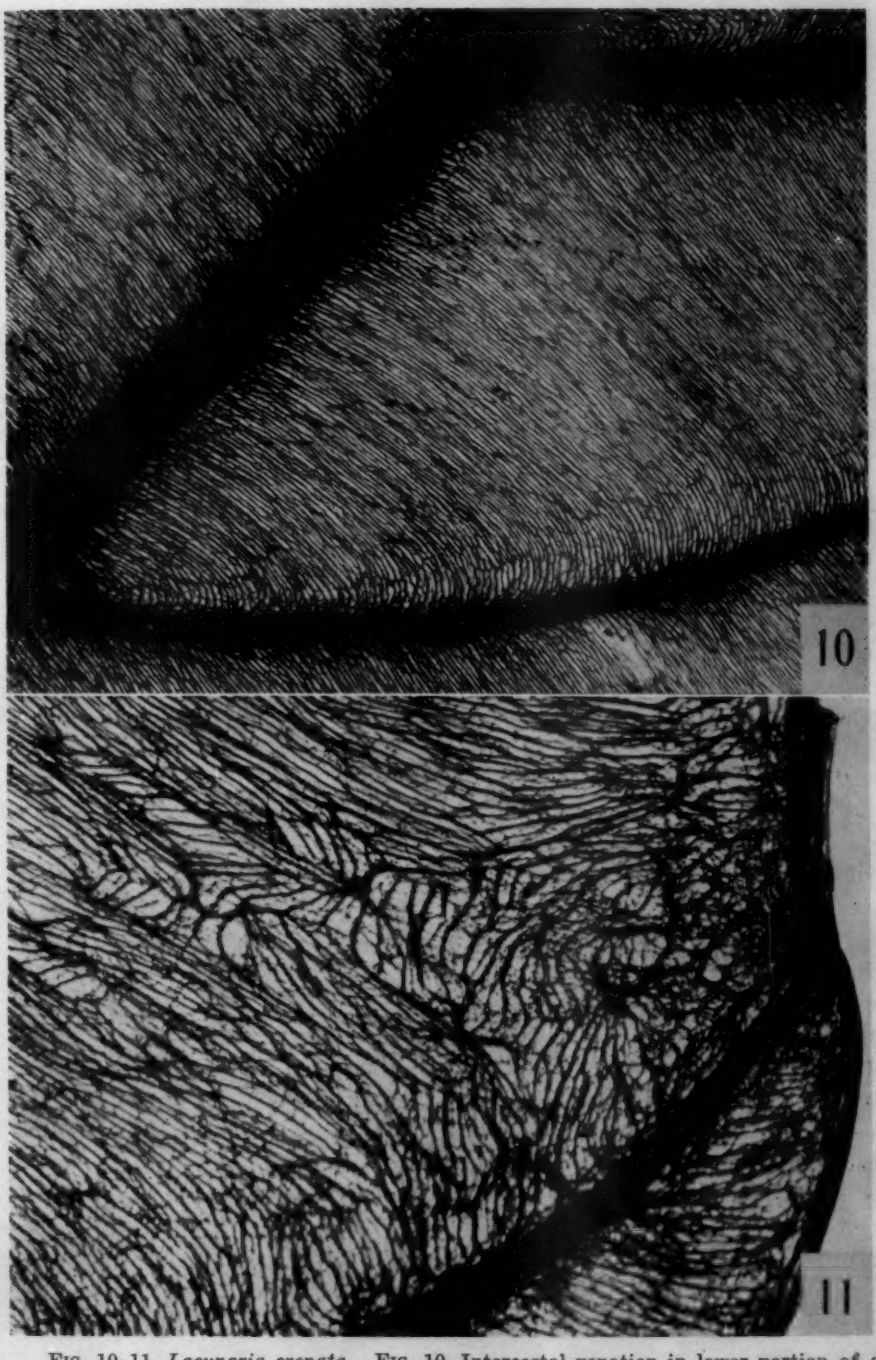


FIG. 10–11. *Lacunaria crenata.* FIG. 10. Intercostal venation in lower portion of a transition-form. Further explanation in text. × 11. FIG. 11. Venation of marginal region of adult type of leaf showing conspicuous idiodromes near terminus of secondary vein. Material collected from tree in Bosqué Municipal, Belém, Brazil. × 25.

is a profuse development of sclerenchyma and numerous "veinlets" consist wholly of fibers.

Adult type of leaf. In order to study as fully as possible the range in pattern of the minor venation, leaves from a series of collections of *L. crenata* were compared. The largest number of leaves of various sizes from a single individual were collected by Dr. Pires from a pistillate tree growing in the Bosqué Municipal in Belém. Collections from other areas in Pará, Amazonas, and Oiapoque in Brazil, and from several different localities in Surinam, provided additional material. In all cases, the intercostal venation consists of extensively anastomosed and dichotomously branched vascular strands together with abundant interspersed fiber strands. Although the true veinlets and strands of fibers are too undulate and irregular in their course to be described as "parallel", the prevailing direction of most of the strands is at right angles to the secondary veins. An exception to this is provided by the somewhat irregular curvature of the veinlets near the point of attachment of the secondary veins to the midrib (fig. 12a).

The most interesting result of the survey of leaves from 22 different collections was the discovery of distinctive areas within the intercostal reticulum characterized by an abrupt change in the course and in the width of spacing of the veinlets (figs. 11–13). These discrete areas fluctuate in size, number and distribution within the lamina. They are entirely absent in the leaves of certain collections (e. g. Ducke 20476, HJBR) and are exceptionally abundant in others. The term "*Idiodrome*" (*idios*, own, private, peculiar—*dromos*, a course) is proposed by the writer as a term to designate an area characterized by a distinctive venation pattern and the adjectival form "*idiodromous*" is suggested as a descriptive term for veinlets whose course differs conspicuously from their neighbors.

The often remarkably discrete nature of the idiodromes in *L. crenata* is clearly shown in figure 12. In this case, a large solitary idiodrome is situated midway between two secondary veins in an intercostal field near the base of the lamina (fig. 12a). When viewed at higher magnification (fig. 12b) the idiodrome is very conspicuous because (1) the constituent veinlets are oriented obliquely and transversely to the prevailing course of the vascular strands bordering the idiodrome and (2) the spaces between the idiodromous veinlets are considerably wider than the average intervascular interval elsewhere in the reticulm. The majority of these idiodromous veinlets contain tracheary elements and only two strands consist exclusively of sclerenchyma.

Further examples of large and distinctive types of submarginal idiodromes are shown in figure 13. One of these idiodromes (fig. 13a) is the largest encountered in this investigation and is further distinguished by the conspicuous dichotomous branching of some of the veinlets and by the

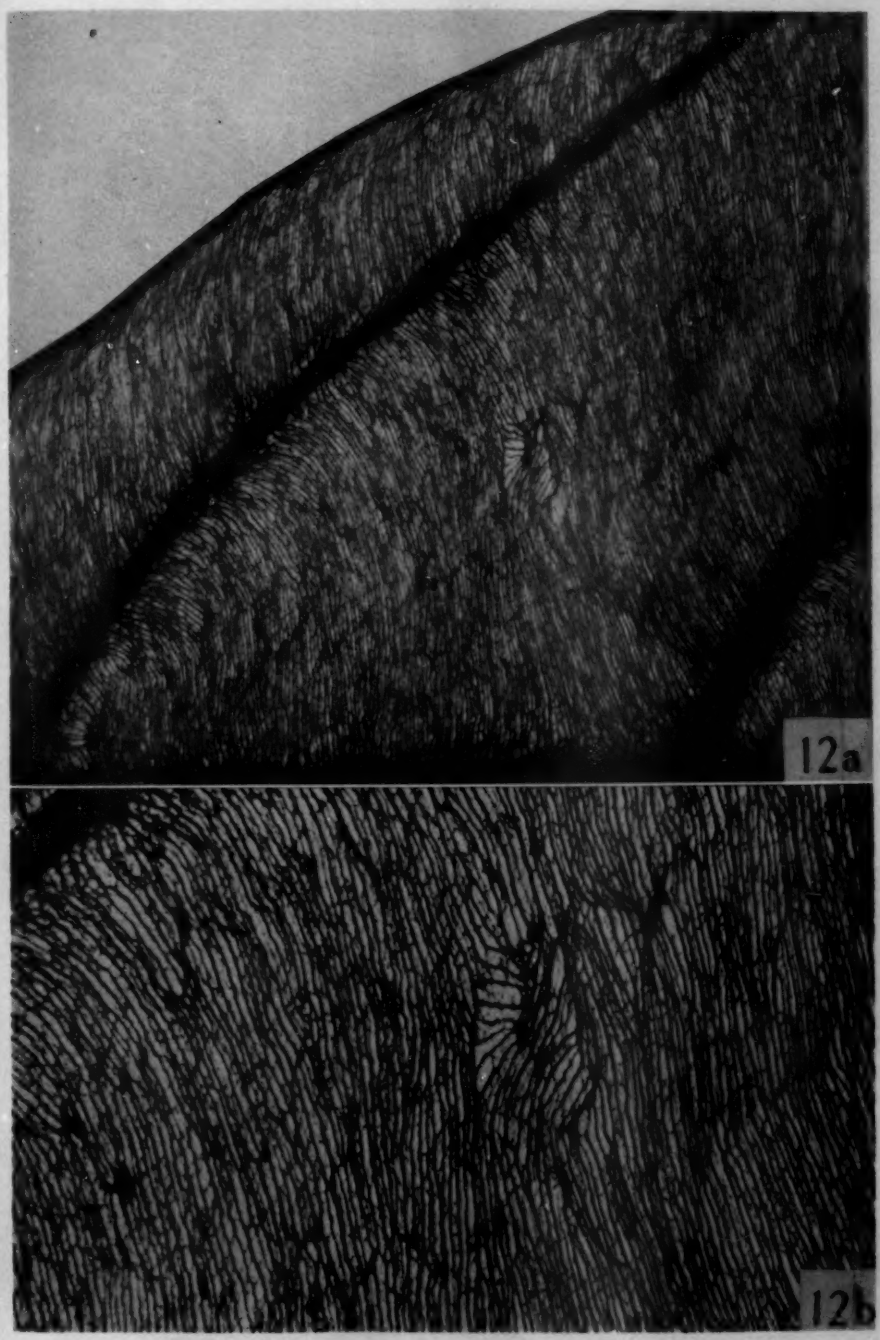


FIG. 12a-b. *Lacunaria crenata*. (Black 49-8619. IAN, UC.). FIG. 12a. Lower portion of a lamina showing a large solitary idiodrome in the middle of the intercostal reticulum. × 19. FIG. 12b. Details of the same idiodrome and neighboring veinlets at higher magnification. Note the transverse-oblique orientation of the widely-spaced veinlets of the idiodrome. × 25.

FIG. 13–14. FIG. 13a-b. *Lacunaria crenata*. Examples of large, distinctive submarginal idiodromes from adjacent intercostal fields of the same lamina. Material collected from tree in Bosqué Municipal, Belém, Brazil. Further description in text. × 39. FIG. 14. *Lacunaria* sp. Portion of the minor venation of a seedling leaf showing a small idiodrome. × 39.

very wide and irregular areoles. Within the latter may be seen the divergent and intrusive tips of numerous fibers. The largest of the idiodromes depicted in figure 13b is very distinctive because it exhibits a striking arcuate pattern. The seven prominent arches are very reminiscent on a small scale, of the intercostal arches in the leaflet of *Touroulia* or the arcuate pattern between the tertiary or intermediate veins in the leaf of *Quiina acutangula* (Foster 1950a, 1950b). Figure 13b also shows, as a relatively unusual feature, a large triangular areole at the left side of the idiodrome. Study at high magnification shows that the base of this areole is marked by a fiber-strand while the two sides consist of tracheid-containing strands.

Idiodromes are also encountered at the margins of the lamina and are likely to be particularly conspicuous near the termination of a secondary vein (fig. 11). Large marginal idiodromes are often bizarre in appearance because of the highly irregular and varied course of the veinlets. Often several series of veinlets, oriented at right angles to one another and to the strands in the surrounding reticulm constitute a wedge-shaped idiodrome which merges inwardly into the normal venation pattern. Figure 11 likewise illustrates the fact that certain idiodromes may be relatively small and consist of only a few transverse or obliquely oriented veinlets; there is of course no sharp boundary between such idiodromes and minor irregularities in the neighboring reticulm.

Idiodromes apparently are very infrequently developed in seedling and transitional leaves and were encountered in only one pinnatifid leaf of *Lacunaria sp.* (fig. 14). Although a complete survey of adequate leaf material of other species of *Lacunaria* has not been attempted, it is worthy of record that small idiodromes, of the type found in *L. crenata*, were observed in the following species: *L. acreana*, Kuhlman 731 (RB); *L. grandifolia*, Froés 22151 (IAN); *L. macrostachya*, Lawrance 537 (F); *L. minor* s/n (RB No. 21098); *L. pulchrinervis*, Ducke s/n (RB, No. 23790); *L. Sampaioi*, s/n (RB No. 23652); *L. Spruceana*, Krukoff 6443 (IAN).

Discussion. The striking difference in leaf-form between seedling and adult plant in *Lacunaria sp.* and *L. crenata* represents an additional example of the remarkable heterophylly which occurs in the Quiinaceae. It is now evident that the duration of the phase marked by the development of the pinnatifid leaves may be relatively short, as in the species of *Lacunaria* described in this paper, or considerably protracted as in *Quiina acutangula*. In this plant, the transition from pinnately lobed to entire leaves does not occur in the seedling stage but is delayed for a number of years. (Foster 1950a p. 160–161, figs. 1–3). The physiological sgnificance, if any, of such marked differences in the extent of the "juvenile phase" in the life of the individual is at present obscure. Speculation on this question would seem premature especially because of our ignorance of the extent and "expres-

sion'' of heterophylly in other species of *Quiina* and *Lacunaria*. In addition to *Q. acutangula*, material kindly provided by Dr. J. Murça Pires shows that *Q. amazonica* A. C. Smith is also conspicuously heterophyllous; in contrast, the seedling leaves of *Q. leptoclada* Tul., *Q. indigofera* Sandw. and *Q. oblanceolata* Sandw. are simple and resemble the adult foliage leaves. Further investigation may possibly reveal intermediate *Lacunaria*-like types of heterophylly in other species of *Quiina*.

From the phylogenetic standpoint it may be significant that the two genera in the Quiinaceae characterized by simple adult leaves i.e. *Quiina* and *Lacunaria*, contain species which develop pinnatifid seedling leaves. The ontogenetic sequence in organogeny of these forms might be interpreted as ''recapitulation'' especially since the two remaining genera, *Touroulia* and *Froesia*, have pinnatifid adult leaves (Foster 1950b). Stebbins (1950 p. 488–492) has recently discussed several examples in dicotyledons where the pinnatifid seedling leaves differ from the adult foliage but resemble the foliar organs of other genera in the same family. He concludes that seedling similarity ''is due to the fact that the genetic factors which alter the apical meristem so that it produces appendages of a more specialized type often begin their action relatively late in development.'' With reference to the Quiinaceae, the evidence from seedling, and juvenile leaves is in favor of a pinnatifid leaf as the primitive or ancestral foliar type in this family.

Several interesting features of the venation patterns observed in the various leaf types in *Lacunaria* deserve emphasis at this point. Among the most distinctive of these features is the erratic occurrence and ultimate disappearance of tertiary veins in the lateral lobes of pinnatifid leaves. In many cases, the lateral lobes are entirely devoid of tertiary veins and the minor venation is very similar to that in small pinnatifid seedling leaves of *Touroulia* (cf. fig. 5 of this paper with Foster 1950b, fig. 4). When tertiary veins develop in *Lacunaria*, they vary widely as to number in a given leaf and as to distribution in the lobes in which they occur (figs. 4, 6, 7). In *Touroulia*, the corresponding veins[2] increase in number and size in passing from the seedling to the adult leaf (Foster 1950b figs. 4–8). But in *Lacunaria*, the gradual reduction of the lateral lobes and their final suppression are accompanied by the disappearance of the tertiary veins. Possibly this is further evidence that the adult leaf in *Lacunaria* has been derived phylogenetically from a pinnatified leaf which had a tertiary vein system. Another interesting feature in *Lacunaria* is the progressive increase in the number of major secondary veins which accompanies the transition from seedling to adult leaf (fig. 3); an entirely comparable elaboration of the

[2] These veins in *Touroulia* and *Quiina acutangula*, with reference to the midrib of the lobe, were designated as ''secondary veins'' by the writer.

major venation likewise occurs in *Quiina acutangula* (Foster 1950a, figs. 1–3).

Finally, the pattern and histology of the minor venation need to be considered briefly. The most unusual feature of the minor venation is the occurrence of *idiodromes*. These areas of distinctive and often bizarre venation pattern were observed in most of the collections of *L. crenata* as well as in a number of other species in the genus (figs. 11–14). What are the factors which induce the development of idiodromes in various parts of a given lamina? There is no evidence that they are "pathological structures" or that they are caused by mechanical injury or the attack of insects. On the contrary, the idiodrome, in the writer's opinion, reflects a local disturbance in the "normal" direction of differentiation of procambium and as such presents a problem of considerable morphogenetic interest. Close inspection of figures 11–14 will show that the veinlets of an idiodrome, despite their erratic course, "merge" with the pattern of veinlets which surround them. This clearly suggests that idiodromes are not formed late in leaf ontogeny but originate during the early phases of vasculation in the lamina. From an histological viewpoint, the minor venation of *Lacunaria sp.* and *L. crenata* is distinguished by the excessive development of fibers which not only diverge intrusively into the mesophyll but which also form massive veinlet-like strands (fig. 8). In striking contrast to *Touroulia* and *Froesia,* however, the foliar veinlets of *Lacunaria* like those of *Quiina,* are devoid of crystalliferous sclereids (Foster 1950b).

SUMMARY

1. In the two species of *Lacunaria* investigated, the early leaves of the seedlings are deeply pinnatifid and quite unlike the simple adult-type of foliage leaf which occurs in all species of this genus.

2. The transition from the pinnatifid to the simple type of leaf consists in the reduction and ultimate suppression of lateral lobes. This is accompanied by a conspicuous increase in the number of secondary veins; these additional veins arise in the terminal and basal regions of the leaves.

3. Tertiary veins, variable in number and degree of development, occur in the lateral lobes of many of the pinnatifid leaves studied. Concomitant with the reduction of the lateral lobes, these tertiary veins disappear and cannot be identified in the lamina of adult leaves.

4. The minor venation of seedling and transition-form leaves consists of two categories of "veinlets," true conducting strands consisting of fiber and tracheary elements and "false veinlets" which are composed wholly of fibers. Most of the true veinlets are unbranched; a low percentage of them dichotomize, one shank commonly ending blindly in the mesophyll the other uniting with the tracheids or fibers of the marginal vein.

5. In the adult leaf, the minor venation consists of extensively anastomosed and dichotomously-branched true veinlets and abundant fiber-strands. One of the most distinctive features of the intercostal reticulum is the erratic occurrence of areas characterized by an abrupt change in the course and in the width of spacing of the veinlets. The term "idiodrome" is proposed to designate an area of this type. A description of various types of marginal and submarginal idiodromes is given and the occurrence of idiodromes in the leaves of a number of species of *Lacunaria* is recorded.

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